Studies with Agents which Influence Acetylcholine Metabolism in Mouse Brain

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Abstract—By using an intravenous single pulse injection of choline-C^{14} into the tail vein of mice and measuring the endogenous levels of choline and acetylcholine, the effects of agents which influence acetylcholine metabolism in mouse brain were studied. The results were consistent with a feedback activation of cholinergic neurons with scopolamine, choline-0-acetyltransferase inhibition with naphthylvinylpyridine, inhibition of acetylcholine release by morphine and cholinesterase inhibition with physostigmine. The interactions of these agents with the cholinergic system further suggests certain characteristics of cholinergic neurons, e.g. that choline-0-acetyltransferase activity is accelerated by a decrease in intracellular acetylcholine levels and retarded by an increase in intracellular acetylcholine levels, that the choline transport mechanism is functioning at maximum capacity under normal physiological conditions and that the synaptic levels of endogenous choline play a role in tracer choline capture.

Introduction

In 1969 Schuberth, et al. (1) introduced a technique for measuring the rate of synthesis of acetylcholine (ACh) in whole mouse brain. In their procedure the rate of radiactive ACh formation in the brain was measured following an intravenous pulse injection of tritium-labelled choline (Ch). After determination of the isotope dilution of the Ch in the brain, the ACh rate of synthesis, *in vivo*, was calculated. We have now applied this technique involving four agents purported to affect central cholinergic neurons in different ways. The agents are the muscarinic cholinergic receptor blocking agent, scopolamine; the choline-0-acetyltransferase inhibitor, naphthylvinylpyridine (2, 3); the cholinesterase inhibitor, physostigmine; and an agent reported to block the release of ACh, morphine (4, 5, 6). The results presented below are consistent with the proposed

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mechanisms of the various agents and help to further define certain characteristics of central cholinergic neurons.

Methods

Administration of Choline-Methyl-C^{14} (Ch-C^{14}). Male CF_1S (Carworth Farms) mice, 18–20 g were used. Each mouse received 5 μc (0.66 μ moles) of Ch-C^{14} chloride (New England Nuclear, specific activity $= 7.6$ μc/μ mole) intravenously in a tail vein. The tracer was dissolved in distilled water and administered in a volume of 0.1 ml with a gas-tight Hamilton syringe through a 30 gauge needle.

Preparation of Brain Samples. The mice were sacrificed at different times after injection by rapid immersion of the whole animal into a Dewar flask containing liquid N_2 for 3.5 seconds. Individual mouse brains were removed as rapidly as possible, dissected on a thermoelectric cold plate maintained at 1–2° C, and frozen in liquid N_2. Where whole brain analysis was performed, the brain was placed directly in liquid N_2. Timing was critical with this method. If the immersion lasted for more than 4 seconds, the surface of the cortex began to freeze and stick to the skull making removal of the tissue difficult. The frozen brains or brain parts were weighed on a Mettler electronic balance and then pulverized in a Thermovac (R) tissue crusher cooled with dry ice and liquid nitrogen. The remainder of the procedure was as described for rats by Saelens *et al.* (7).

Determination of ACh *and* Ch. ACh and Ch were determined by the enzymatic method of Feigenson and Saelens (8) using the modifications recommended by Saelens *et al.* (9).

Statistical Analysis. The disposition of Ch-C^{14}, and the levels of Ch and ACh were determined in different groups of mice. In order to obtain the appropriate parameters, the product of two independent variables were analyzed by the method of Goodman (10). At least 5–10 mice were used in all measurements.

Agents. Test agents were dissolved in distilled water, and administered subcutaneously, or intraperitoneally, in a volume of 0.1 ml/10 g of body weight or intravenously in a volume of 0.05 ml/10 g of body weight. Mice received food and water *ad libitum* up until the time of sacrifice.

The agents studied were scopolamine hydrobromide (Merck), naphthylvinylpyridine (Aldrich), physostigmine sulfate (Merck) and morphine sulfate (Merck).

Results and Discussion

Control Studies: The Schuberth model for estimating the rate of synthesis of ACh is essentially a single closed compartment model. To be valid, the level of

precursor tracer should be reasonably constant during the time course of product tracer formation. Further, product tracer formation should be linear over the same time course and should be as far below the equilibrium point as possible. Figure 1 shows that the first 2 criteria were satisfied for the first 45–60 seconds after intravenous administration of the Ch-C[14]. Maximal Ch-C[14] levels were found in the whole mouse brain within 5 seconds. The total dpms of Ch-C[14]
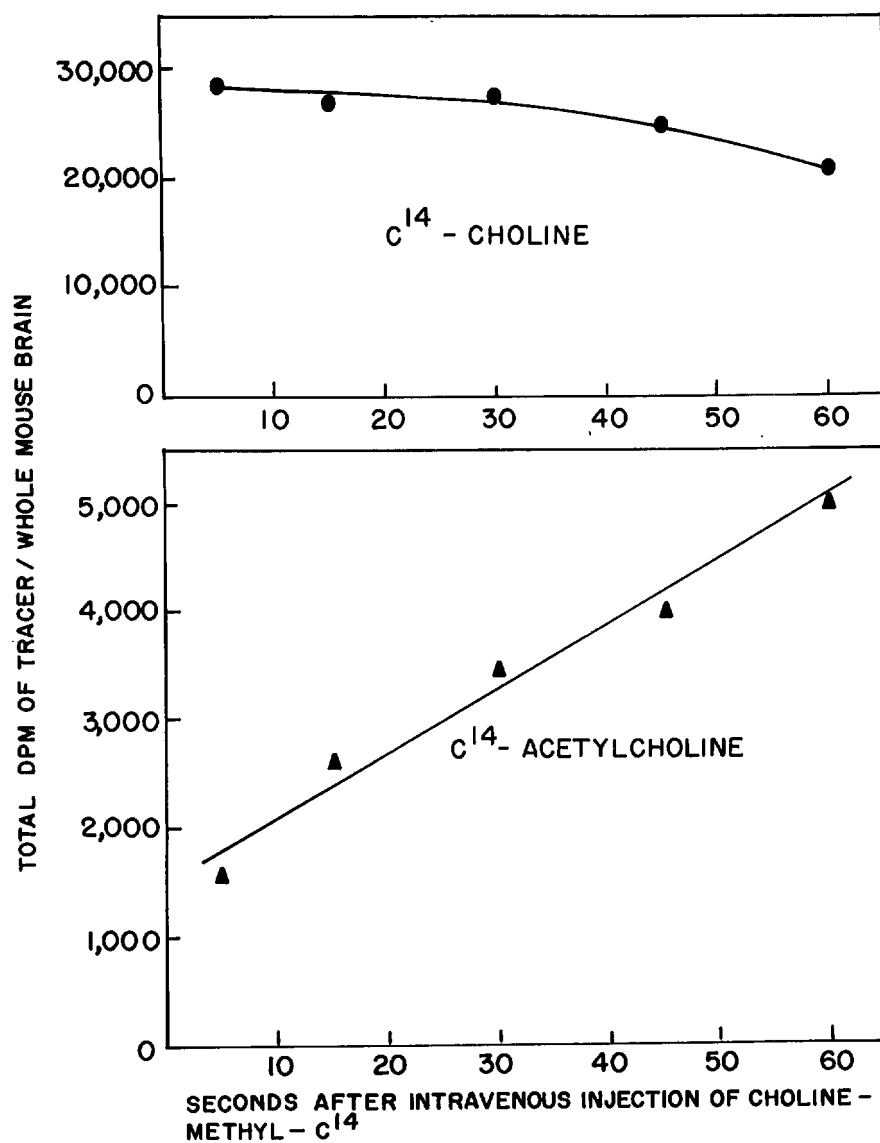

FIG. 1

remained relatively constant for 45–60 seconds and during that time the ACh-C^{14} levels increased in a linear fashion.

The question of equilibrium necessitated looking at the tracer product-precursor relationship over a longer time course. Mice were injected with Ch-C^{14} intravenously and sacrificed at different time intervals up to 60 minutes. The ACh-Ch^{14}/Ch-C^{14} ratio vs. time is shown in Figure 2 for various parts of mouse brain and whole mouse brain. In all cases, the ratio of ACh-^{14} to Ch-C^{14} rose rapidly, peaked between 5 and 15 minutes and fell slowly over the remainder of the time course. The absolute amounts of ACh-^{14} and Ch-C^{14}, meanwhile, fell continuously throught the time course (data not shown). These data suggested that the model behaved as a single closed compartment system only for the first
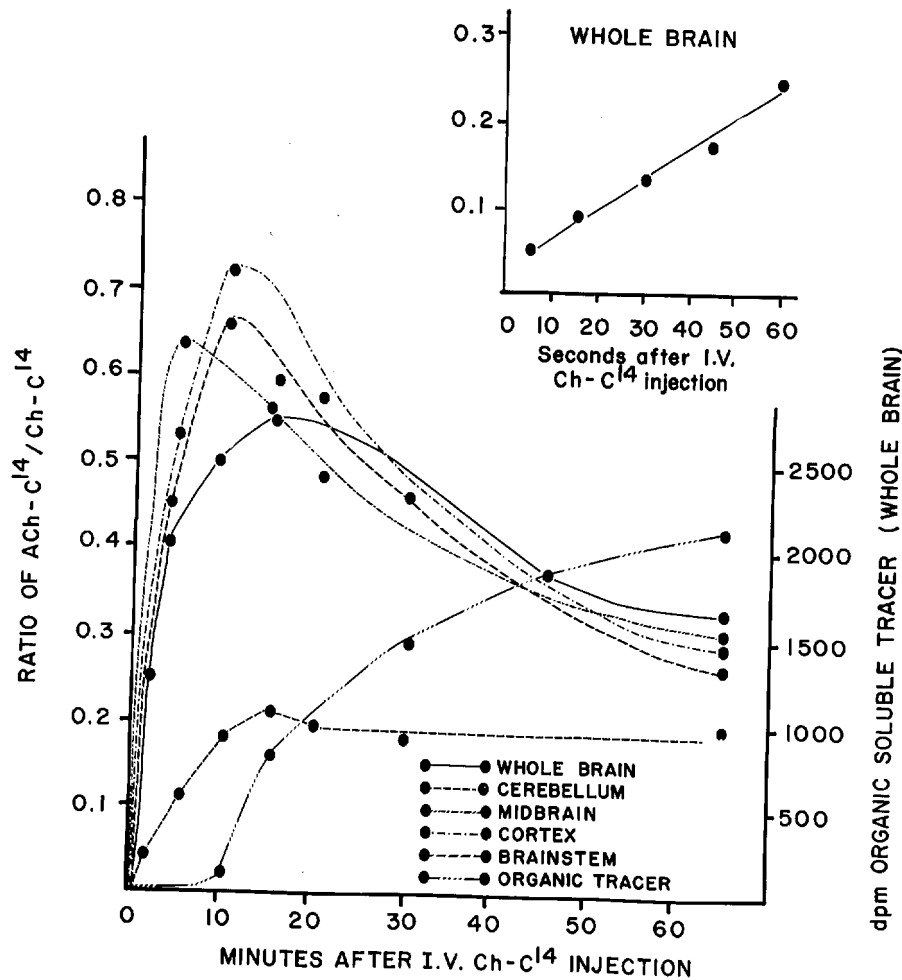

FIG. 2

few minutes after administration of the tracer. $ACh\text{-}C^{14}/Ch\text{-}C^{14}$ ratios obtained at one minute or less were clearly below the peak ratios and therefore data generated using this time interval satisfied the criteria of being below equilibrium for product tracer formation. When the pharmacological agents were used, the mice were always sacrificed 45 seconds after administration of the $Ch\text{-}C^{14}$ and 30 minutes after administration of the agent.

As a working hypothesis, it is suggested that the tracer was equilibrating with a part of the free Ch pool which was readily available for ACh synthesis during the rise of the ratio curve, and the peak and fall were the result of the tracer beginning to equilibrate with other parts of the Ch pool. This interpretation is supported by the delayed appearance of organic soluble tracer (in the acetone-chloroform extract usually discarded when measuring endogenous ACh and Ch). If this hypothesis is correct, then the peaks in the $ACh\text{-}C^{14}/Ch\text{-}C^{14}$ ratio curves should be reasonable, albeit conservative, estimates of the ratio between endogenous ACh and the portion of the Ch pool suggested to be available for ACh synthesis. The peak ratios, therefore, allowed an indirect estimate of the size of the available free Ch pool (AF Ch) using the following equation:

$$AF\ Ch = \left[\frac{Ch - C^{14}}{ACh - C^{14}} \right]_{at\ peak} \times ACh\ (endogenous)$$

Using this indirect technique, the sizes of the AF Ch pools and the synthesis rates of ACh in the whole brain, cortex, midbrain, brainstem and cerebellum were estimated and are shown in Table I. Under these conditions, it was estimated that the stores of ACh in mouse brain would be replaced every 2–3 minutes.

Using the direct technique, it should be noted that the endogenous brain Ch levels of control animals shown in Table 2 were approximately twice the AF Ch estimated above. Recent investigators, particularly Ewetz *et al.* (11), suggest that post-morten increases in Ch levels due to catabolic destruction of Ch containing compounds in the brain, are very difficult to avoid. It is possible that, despite the precautions taken, the Ch levels in Table II include some Ch originating from such sources. For this reason, when the single closed compartment calculations for rate of synthesis of ACh were applied to the data presented here the results were expressed as "apparent rate of synthesis."

Scopolamine: At 10 mg/kg i.p., scopolamine caused a 50 % decrease in endogenous ACh levels. However, the amount of $ACh\text{-}C^{14}$ found in the brain at 45 seconds was actually slightly increased. Thus, the specific activity of the reduced endogenous ACh pool was 2.4 times the specific activity of the corresponding control ACh pool. This dose of scopolamine also caused a statistically significant decrease in the endogenous Ch levels and a slight decrease in the 45 second $Ch\text{-}C^{14}$ levels. All the data with scopolamine is consistent with a feedback activation of cholinergic neurons resulting from cholinergic receptor blockade. The conversion of $Ch\text{-}C^{14}$ to $ACh\text{-}C^{14}$ was clearly accelerated as illustrated by the large increase in ACh specific activity but the reduction in the

endogenous Ch pool counterbalanced the significantly higher ACh-C^{14}/Ch-C^{14} ratio resulting in no change in the apparent synthesis of ACh. The reduction in ACh levels caused by scopolamine is most likely a matter of synthesis being unable to keep up with the drug-induced facilitation of release. These results suggest 2 characteristics of cholinergic neurons. First, the choline-0-acetyl-transferase activity is accelerated by a decrease in ACh levels and/or Ch levels, the former, alone, being the most logical. Second, Ch capture is not accelerated by the decrease in ACh or Ch levels suggesting that the Ch transport mechanism is functioning at maximum capacity under normal physiological conditions.

TABLE I

Estimates of the AFCholine Pools and Rates of Synthesis of Acetylcholine in Various Brain Areas of Control Mice

Area	Mean ACh[a] mμ Moles/g	$\left[\dfrac{ACh - C^{14}}{Ch - C^{14}}\right]$[b] peak	Estimate AF Ch[c] mμ Moles/g	$\left[\dfrac{ACh - C^{14}}{Ch - C^{14}}\right]$ 1 minute	Estimates of Rate of Synthesis of ACh mμ Moles/ g/minute
Whole Brain	13.6	0.550	24.7	0.263	6.50
Cortex	12.5	0.635	19.6	0.339	6.64
Midbrain	18.5	0.720	25.6	0.244	6.25
Brainstem	19.7	0.661	29.8	0.189	5.63
Cerebellum	6.9	0.201	33.6	0.040	1.34

[a] ACh = Acetylcholine endogenous.

[b] $\left[\dfrac{ACh - C^{14}}{Ch - C^{14}}\right]_{peak}$ = the peak ratios from Figure 2.

[c] AF Ch—Available Free Choline—the hypothetical choline pool suggested to be available for acetylcholine synthesis.

Naphthylvinylpyridine (NVP). The choline-0-acetyltransferase inhibitor, NVP, caused a dose related increase in endogenous ACh levels. The *a priori* expectation would be just the opposite, *i.e.* a decrease in endogenous ACh levels. However, others have also failed to demonstrate ACh depletion with NVP (2, 3). The increase in endogenous ACh was associated with a dose related decrease in the 45 second ACh-C^{14} levels which was statistically significant at 40 mg/kg i.v. The specific activity of ACh in NVP treated animals also showed a dose related decrease which dropped to 40 % of control at 40 mg/kg i.v. Thus, NVP, at reasonable doses, was quite capable of depressing the conversion of Ch-C^{14} to ACh-C^{14} but was incapable of reducing storage levels of ACh. The Ch transport

TABLE II

Effect of Various Agents on Acetylcholine and Choline Levels and the Disposition of Methyl-C14 Choline

Parameter	Control	Scopolamine 10 mg/kg i.p.	Control	NVP 10 mg/kg i.p.	NVP 20 mg/kg i.p.	NVP 40 mg/kg i.p.	Control	Morphine 1 mg/kg s.c.	Morphine 3 mg/kg s.c.	Morphine 10 mg/kg s.c.	Control	Physostigmine 0.1 mg/kg s.c.
ACh (mμ M/g)	18.8 ± 1.5	9.3 ± .6*	16.2 ± 1.3	21.8 ± 1.6*	25.2 ± 1.8*	24.2 ± .8*	20.8 ± 1.6	17.0 ± .7	19.7 ± 0.8	22.0 ± 1.1	19.5 ± 2.3	25.6 ± 3.0
ACh-C14 @ 45 Seconds (dpm/g/10⁻⁴)	1.58 ± .04	1.89 ± .16	1.66 ± .19	1.33 ± .13	1.20 ± .09	0.97 ± .12*	1.08 ± .05	1.00 ± .13	1.37 ± .08	0.92 ± .03	1.00 ± .06	0.47 ± .04*
Specific Activity of ACh @ 45 Seconds (dpm/mμ M)	844 ± 60	2037 ± 215*	1021 ± 147	612 ± 76*	476 ± 48*	399 ± 52*	523 ± 45	591 ± 77	689 ± 47	419 ± 23	513 ± 64	183.6 ± 70*
Ch (mμ M/g)	56.4 ± 6.9	46.1 ± 3.1*	65.1 ± 6.4	60.6 ± 5.6	66.5 ± 9.1	62.3 ± 4.7	68.1 ± 4.6	57.8 ± 1.7	60.9 ± 4.0	62.1 ± 3.9	54.3 ± 5.3	51.7 ± 4.6
Ch-C14 @ 45 Seconds (dpm/g/10⁻⁴)	5.47 + .78	5.04 ± .25	8.15 ± .59	7.65 ± .12	8.98 ± .24	8.43 ± .87	7.04 ± .23	8.54 ± .51*	11.43 ± .33*	12.58 ± .25*	6.18 ± .70	8.74 ± .73*
Specific Activity of Ch @ 45 Seconds (dpm/mμ M)	969 ± 74	1093 ± 83	1252 ± 98	1262 ± 47	1350 ± 53	1354 ± 61	1034 ± 75	1480 ± 90*	1874 ± 132*	2024 ± 89	1138 ± 84*	1638 ± 133*
ACh-C14/Ch-C14 @ 1 Minute	0.30	0.38*	0.25	0.25	0.20	0.15*	0.21	0.15*	0.16*	0.10*	0.16	0.05*
Apparent Rate of Synthesis of ACh (mμM/g/Min)	16.8 ± 1.1	17.4 ± 1.9	16.3 ± 1.2	15.1 ± 1.2	13.3 ± 1.7	9.4 ± 0.9*	14.0 ± 1.2	8.9 ± 0.8*	9.7 ± 1.0	6.1 ± 0.5*	11.7 ± 1.3	3.7 ± 2.1*

*$p \leq .05$

mechanism is also apparently independent of choline-0-acetyltransferase activity as NVP clearly depressed the conversion of Ch-C^{14} to ACh-C^{14} but had no effect on the capture of Ch-C^{14} from the bloodstream. Therefore, there is no evidence here, that choline-0-acetyltransferase is a Ch acceptor inside the nerve ending necessary for the Ch transport mechanism to function properly. NVP did cause a significant reduction in the apparent rate of synthesis at 40 mg/kg i.v. Why the ACh levels went up instead of down is still unclear.

Morphine: In a dose range of 1–10 mg/kg s.c. morphine was noteably ineffective in altering all parameters involving ACh but dramatically increased the 45 second Ch-C^{14} levels without altering endogenous Ch levels. The apparent rate of ACh synthesis was significantly decreased by all doses of morphine tested but clearly this was a consequence of increased Ch-C^{14} capture. There is considerable evidence that morphine impairs the release of ACh in the brain (4, 5, 6). There is also some evidence that ACh released into the synaptic cleft is hydrolyzed by cholinesterase and the resulting Ch transported back into the cholinergic nerve ending (*e.g.* 7). Morphine is almost doubled in the 45 second Ch-C^{14} capture at 10 mg/kg s.c. It is tempting to speculate that these 3 events are interrelated. The tracer pulse most probably passes by the brain as a bolus where the peak specific activity is exposed to the nervous tissue for a very short period of time. Once into the extracellular space, including cholinergic neuron synapses, it is taken up by the Ch transport mechanism into the neuron. At this juncture, the tracer Ch-C^{14} is suggested to undergo some amount of isotope dilution from the non-radioactive Ch hydrolyzed from the non-radioactive ACh being continuously released during transmission. If morphine impairs the release of ACh, the tracer should undergo a lesser amount of isotope dilution as the source of dilution, the released ACh, is diminished. It is suggested that the cholinergic nerve endings are not taking up a larger quantity of Ch but rather are taking up the same quantity of Ch which has a higher proportion of Ch-C^{14}. This hypothesis is consistent with the fact that the endogenous Ch levels do not change under the influence of morphine.

Physostigmine: The above hypothesis regarding morphine and Ch-C^{14} capture was challenged by reducing the availability of synaptic Ch in a different way. Physostigmine does not impair the release of ACh (2), but does prevent the hydrolysis of ACh to Ch in the synapse. With 0.1 mg/kg s.c. of physostigmine, again, the 45 second Ch-C^{14} levels were substantially higher than corresponding controls. It was also of interest to note that physostigmine significantly decrease the 45 second ACh-C^{14} levels and specific activity of ACh supporting the contention of Kaita and Goldberg (12) that feedback inhibition by intracellular ACh plays a role in ACh synthesis. The effects of physostigmine on ACh related parameters also clearly distinguishes it from morphine.

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